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Calmodulin interacts with the cytoplasmic tails of the parathyroid hormone 1 receptor and a sub-set of class b G-protein coupled receptors

Matthew J. Mahon*, Masako Shimada

Endocrine Unit, Massachusetts General Hospital, 50 Blossom Street, Wellman 501, Boston, MA 02114-2696, USA

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Abstract Parathyroid hormone (PTH) binds to its receptor (PTH 1 receptor, PTH1R) and activates multiple pathways. The PTH1R, a class b GPCR, contains consensus calmodulin-binding motifs. The PTH1R cytoplasmic tail interacts with calmodulin in a calcium-dependent manner via the basic 1-5-8-14 motif. Calcium-dependent calmodulin interactions with the cytoplasmic tails of receptors for PTH 2, vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide, corticotropin releasing hormone, calcitonin, and the glucagon-like peptides 1 and 2 are demonstrated. The cytoplasmic tails of the secretin receptor and the growth hormone releasing hormone receptor either interact poorly or not at all with calmodulin, respectively. Fluphenazine, a calmodulin antagonist, enhances PTH-mediated accumulation of total inositol phosphates, suggesting that calmodulin regulates signaling via phospholipase C.

Keywords: Calmodulin; Receptor, Parathyroid hormone type 1; Receptor, G-protein coupled

1. Introduction

The parathyroid hormone 1 receptor (PTH1R) is a class b G-protein coupled receptor (GPCR) that is bound and activated by two prominent ligands, parathyroid hormone (PTH), an endocrine factor involved in mineral ion homeostasis, and PTH-related protein (PTHrP), an autocrine/paracrine factor involved in development [1]. The PTH1R couples to Gs, Gq/11 and Gi/o and signals via adenylyl cyclase (AC) and phospholipase C (PLC) [2,3]. Cell-specific responses elicited by PTH or PTHrP are undoubtedly dependent upon the complement of available G proteins, however, recent evidence demonstrates that non-G protein, cytosolic factors bind to and regulate the activity of the PTH1R. The sodium-hydrogen exchanger regulatory factors (NHERFs) 1 and 2 bind to the carboxyl terminus of the PTH1R and enhance signaling via PLC [2,3].

Calmodulin (CaM) is a conserved, ubiquitous, calcium sensing protein that binds and regulates many intracellular proteins, including cytoskeletal elements, ion channels, kinases, phosphatases and enzymes associated with GPCR signaling, such as AC, phosphodiesterase and PLC [4]. Two generalized CaM-binding motifs exist. The Ca^{2+}-independent CaM-binding site based on myosin interactions contains the consensus sequence shown in Table 1 and is referred to as the IQ-motif named for the first two conserved amino acid residues [5]. The Ca^{2+}-dependent CaM-binding motif is more divergent and is primarily based on positions of conserved hydrophobic residues flanked by basic residues [5]. These motifs are referred to as either the basic 1-5-8-14 (Table 1) or basic 1-5-10. Here, we show that the PTH1R and a sub-set of class b GPCRs bind to CaM in a Ca^{2+}-dependent manner via determinants in the cytoplasmic, carboxy-terminal tails.

2. Materials and methods

2.1. Reagents

The pGex vector and glutathione–Sepharose were from Pharmacia/Amersham. The bacterial expression vector p30, S-protein-HRP and rapid S-tag assay kit were from Novagen. The Ni^{2+}-chelate column (Ni–NTA) was purchased from Qiagen. ECL Western lightning reagent was from Perkin–Elmer. Secondary-antibodies conjugated to Alexa Fluor 546 were obtained from Molecular Probes. *Pfu* polymerase was purchased from Stratagene and restriction enzymes were from Promega. Flag-specific monoclonal antibodies and general chemicals were from Sigma.

2.2. GST pull-down assay

The full-length cytoplasmic, carboxy-terminal tail (C-tail) of the PTH1R (amino acids 463–591) was cloned into the p30 vector generating an N-terminal his_6- and S-tag. PTH1R C-tail amino-terminal deletions of 5 (amino acids 468–591), 10 (473–591), 15 (478–591) and 20 (483–591) residues and the various point mutations were generated using PCR-directed mutagenesis. Full-length carboxy-terminal tails of the PTH 2 receptor (PTH2R, amino acids 417–550), vasoactive intestinal peptide receptor (VIP1R, 392–457), pituitary adenylate cyclase activating peptide receptor (PACPR, 461–525), corticotropin releasing hormone 1 receptor (CRF1R, 367–415), calcitonin receptor (CALTR, 396–482), the glucagon-like peptide receptors 1 (GLP1R, 406–463) and 2 (GLP2R, 440–553), secretin receptor (SECRR,

*Corresponding author. Fax: +1 617 726 7543.
E-mail address: mahon@helix.mgh.harvard.edu (M.J. Mahon).

Abbreviations: PTH, parathyroid hormone; PTHrP, parathyroid hormone related protein; PTH1R, parathyroid hormone 1 receptor; GPCR, G-protein coupled receptor; CaM, calmodulin; PLC, phospholipase C; AC, adenylyl cyclase; PTH2R, PTH 2 receptor; PACPR, pituitary adenylate cyclase activating peptide receptor; VIP1R, vasoactive intestinal peptide 1 receptor; CRF1R, corticotropin releasing hormone 1 receptor; CALTR, calcitonin receptor; GLP1R and GLP2R, glucagon-like peptide receptors 1 and 2; SECRR, secretin receptor; GHRHR, growth hormone releasing hormone receptor; OK, opossum kidney cells

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Table 1
CaM-binding motifs in the PTH1R

Type	Motif
IQ	(IVL)QxxxRxxxx(RK)xx(FILVWY)
PTH1R	463[a] – NGE**VQ**AEI**R**KSWS**R**WT**L**ALDFKRKA – 487[a]
1-5-8-14	(FILVW)xxx(FAILVW)xx(FAILVW)xxxxx(FILVW)
PTH1R	463[a] – NGEVQAE**I**RKS**W**SR**W**TLALD**F**KRKA – 487[a]

[a]Amino acid position in rat PTH1R.

392–449) and the growth hormone releasing-hormone receptor (GHRHR, 376–419) were cloned into the p30 vector using PCR. Full-length CaM was cloned into the pGex vector generating a fusion with glutathione *S*-transferase (GST-CaM). All plasmid constructs were verified by sequencing using an ABI Prism sequencer.

The p30-C-tails and the GST-CaM were expressed in BL21-DE3-pLysS *Esherichia coli* strain. The C-tails were partially purified using immobilized metal affinity chromatography using Ni–NTA from Qiagen, following the manufacturer's protocols. Protein concentrations were determined using the S-tag rapid assay kit (Novagen). GST alone or GST-CaM were coupled to glutathione–Sepharose and mixed with the above C-tail preparations (100 nM) in a buffer containing 25 mM HEPES, pH 7.4, 20% glycerol, 150 mM NaCl, 1 mM dithiothreitol, a protease inhibitor cocktail (Sigma) and 10 mg/ml of a crude bacterial lysate for 4 h at 4 °C. The presence or absence of either 1 mM EGTA or 1 mM $CaCl_2$ is indicated in the figures. The GST-CaM/C-tail complex was eluted with 5 mM glutathione and analyzed by SDS–PAGE and blotting using S-protein-HRP and enhanced chemiluminescence.

2.3. Immunohistochemistry

CaM was cloned into a pcDNA3.1 vector (Invitrogen) that places an N-terminal flag epitope on the full-length cDNA (flag-CaM). The yellow fluorescent protein was cloned in-frame with the PTH1R (PTH1R-YFP), as previously described [5]. Opossum kidney (OK) cells cultured in DMEM/F12 plus 10% fetal bovine were transiently transfected with flag-CaM and PTH1R-YFP using FuGene 6 (Roche). The cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized and blocked with 5% non-fat milk in PBS containing 0.1% Triton X-100, and probed with flag-specific antibodies and secondary antibodies conjugated with Alexa Fluor 546. Images were acquired using a Radiance 2000 confocal microscope and the associated software (BioRad).

2.4. Determination of inositol phosphates

HEK293 cells cultured in DMEM plus 10% fetal bovine serum were plated in 12-well plates and transiently transfected with the PTH1R using FuGene 6. Analysis of total inositol phosphates was as previously described [6].

3. Results and discussion

Rhoads and Friedberg [5] reported that the PTH1R contains a conserved IQ CaM-binding site (Table 1). The focus of this report was to survey potential CaM-binding partners and thus direct interactions between the PTH1R and CaM were not investigated. Using the GST pull-down assay, GST-CaM displays a robust interaction with carboxy-terminal tail (C-tail) of the PTH1R (Fig. 1). The IQ-binding motif found in the PTH1R generally mediates Ca^{2+}-independent interactions with CaM. Unexpectedly, the PTH1R C-tail binds to CaM in a Ca^{2+}-dependent manner (Fig. 1).

Deleting amino acids from the N-terminus of the C-tail by increments of five demonstrates that CaM binds to a region located between amino acids 473 and 478 of the PTH1R (Fig. 2). The consensus IQ motif in the PTH1R between

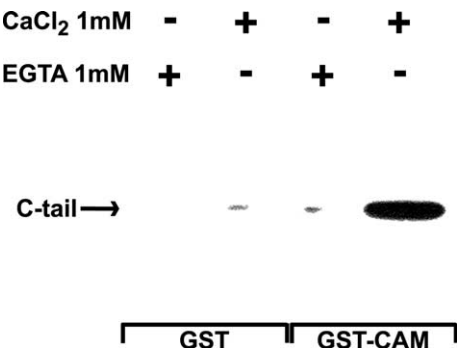

Fig. 1. CaM binds to the carboxy-terminal tail of the PTH1R in a Ca^{2+}-dependent manner. GST alone or GST fused to CaM were interacted with the PTH1R C-tail (100 nM), as described in Section 2, in the presence of either 1 mM $CaCl_2$ or 1 mM EGTA, as indicated. Interactors were eluted with glutathione and blotted with S-protein-HRP.

amino acids 466 and 479 overlaps the CaM-binding region demonstrated in Fig. 2. However, deleting 5 amino acids from the N-terminus, which has no effect on CaM binding, removes the IQ portion of this motif. This finding suggests that despite the overlap CaM does not bind to the PTH1R C-tail via the IQ motif, which is also consistent with the Ca^{2+}-dependence for this interaction. The deletion analysis demonstrates that the SWSRW sequence (amino acids 473–477) is required for the CaM interaction. Individual alanine substitutions for each residue of the SWSRW sequence on the C-tail, however, did not inhibit interactions with CaM (Fig. 2).

The inability of the alanine mutagenesis scan through the CaM-binding domain to block interactions with the C-tail suggests that alanine substitutions are tolerated. Notably, the CaM interaction domain on the PTH1R C-tail nearly matches the basic 1-5-8-14 binding motif (Table 1). The tryptophan residues at amino acids 474 and 477 of the PTH1R correspond to positions 5 and 8 in the basic 1-5-8-14 motif (Table 1). Importantly, alanines in positions 5 and 8 are acceptable residues for the consensus-binding motif, which could explain why alanine substitutions in these positions did not block CaM binding (Fig. 2). Furthermore, the R476A mutation, which targets the 4th position of the IQ motif, has no effect on CaM binding (Fig. 2). These findings, combined with the fact that this interaction is Ca^{2+}-dependent, strongly suggest that CaM binds to the PTH1R C-tail via the basic 1-5-8-14 motif. Sequence alignments of the class b GPCRs reveals that the CaM-binding region is semi-conserved (Table 2).

The C-tails of the class b receptors shown in Table 2 were expressed in bacteria and interactions with GST-CaM assessed. As shown in Fig. 3, CaM displays robust, Ca^{2+}-dependent interactions with the C-tails of the PTH2R, PACPR, VIP1R, CRF1R, CALTR, and GLP1R and GLP2R. The C-tail of the SECRR interacts poorly with CaM, while the C-tail of the GHRHR does not interact at all (Fig. 3). The C-tails of PACPR, VIP1R and CRF1R are expressed and purified as fragments, which is more than likely due to conflicts in mammalian codon usage in bacteria. However, all of these fragments interact with GST-CaM in a Ca^{2+}-dependent manner, which also confirms that the interaction domains are on N-terminal portions of these C-tails.

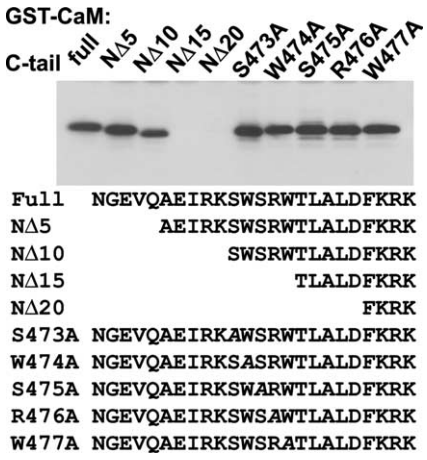

Fig. 2. CaM interacts with N-terminal portions of the PTH1R C-tail. The PTH1R C-tail (full), N-terminal amino acid deletions of the C-tail of 5 (NΔ5), 10 (NΔ10), 15 (NΔ15) and 20 (NΔ20) and the following alanine substitutions of the C-tail, S473A, W474A, S475A, R476A and W477A (indicated by italics), were interacted with GST-CaM in the presence of 1 mM $CaCl_2$. Interactors were eluted and blotted with S-protein-HRP.

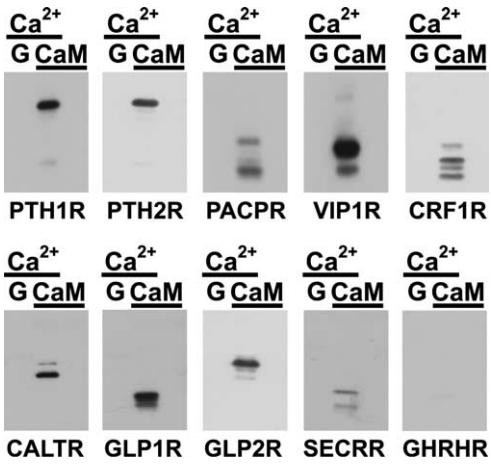

Fig. 3. A sub-set of class b GPCRs interacts with CaM. GST alone (G; 1st lane of each panel) or GST-CaM (CaM; 2nd and 3rd lanes of each panel) were interacted with the C-tails (100 nM) of the class b GPCRs shown in Table 2 in either the presence of 1 mM $CaCl_2$ (1st and 2nd lanes of each panel) or 1 mM EGTA (3rd lane of each panel). Interactors were eluted and blotted with S-protein-HRP.

Table 2
Sequence alignment of class b GPCRs

Receptor[a]	Sequence[b]
PTH1R	VQAEIRKSWSRWTLALDFKRKA
PTH2R	VQAEVKKMWSRWNLSVDWKRTP
PACPR	VQAEIKRKWRSWKVNRYFAVKF
VIP1R	VQAELRRKWRRWHLQGVLGWNP
CRF1R	VRSAIRKRWRRWQDKHSIRARV
CALTR	VQGALKRQWNQYQAQRWAGRRS
GLP1R	VQMEFRKSWERWRLERLNIQRD
GLP2R	VKAELRKYWVRFLLARHSGCRA
SECRR	VQLEVQKKWRQWHLQEFPLRPV
GHRHR	VRTEISRKWHGHDPELLPAWRT

[a]PTH 2 receptor (PTH2R), pituitary adenylate cyclase activating peptide receptor (PACPR), vasoactive intestinal peptide 1 receptor (VIP1R), corticotropin releasing hormone 1 receptor (CRF1R), calcitonin receptor (CALTR), glucagon-like peptide receptors 1 (GLP1R) and 2 (GLP2R), secretin receptor (SECRR) and growth hormone releasing hormone receptor (GHRHR).
[b]Amino acids 466–487 of rat PTH1R.

Sequence differences between the PTH1R and the receptors that displayed weak interactions with CaM (i.e., SECRR and GHRHR) should highlight critical binding determinants for this interaction. First, the SECRR has a glutamine at amino acid 471 relative to the PTH1R sequence instead of an arginine, which is conserved in the IQ-binding motif (Table 1). Second, the GHRHR has a histidine substitution for the conserved tryptophan at amino acid 477. Both SECRR and GHRHR contain a proline instead of a phenylalanine at amino acid 483 of the PTH1R. Using the SECRR and GHRHR as guides, a series of point mutations were incorporated into the PTH1R C-tail and interactions with CaM assessed.

An alanine substitution for the conserved glutamine at amino acid 467 (Q467A) and glutamic acid (R471E) or glutamine (R471Q) substitutions for arginine at position 471 do not affect CaM interactions (Fig. 4). These mutations target conserved positions of the IQ motif (Table 1). Thus, CaM binding to these mutants provides more evidence that these interactions are not mediated by the IQ motif. Incorporating histidine for the tryptophan at position 477 or a proline substitution for the phenylalanine at 483 of the PTH1R also does not block CaM interactions. These findings were unexpected because these mutations, which are based on the GHRHR sequence (Table 2), are key residues in the 8 and 14 positions of the basic 1-5-8-14 motif (Table 1).

Double point mutations incorporating alanines for the two conserved tryptophan residues at positions 474 and 477 (CTWW) markedly inhibit CaM binding (Fig. 4). The double point mutation, R471Q/F483P (CTQP) based on the SECRR sequence, did not affect CaM binding (Fig. 4). Conversely, following the GHRHR sequence, the double mutation, W477H/F483P (CTHP), completely blocks CaM interactions with the PTH1R C-tail (Fig. 4). Combined, these findings reveal that the 5, 8 and 14 positions of the basic 1-5-8-14 motif are critical determinants for the CaM/PTH1R interaction. Furthermore, these data also demonstrate that the CaM/PTH1R interaction occurs through multiple contacts considering that double point mutations are required to effectively block this association.

The PTH1R localizes to actin-rich, apical patches of opossum kidney cells, complexes that are assembled in part by the sodium-hydrogen regulatory factor 1 (NHERF-1) [3]. Opossum kidney cells were transiently transfected with the PTH1R tagged with the yellow fluorescent protein (PTH1R-YFP) and CaM tagged with the flag epitope. Confocal microscopic analysis reveals that the PTH1R and CaM co-localize in the apical patches of opossum kidney cells (Fig. 5). Interestingly, CaM localized to these apical structures in only 20% of the transfected cells, suggesting a dynamic interaction between the PTH1R and CaM in these cellular compartments. The effect of fluphenazine, a CaM antagonist, on PTH signaling via the PLC pathway was investigated. Fluphenazine increases PTH elicited increases of total inositol phosphates (Fig. 6), suggesting that CaM binding to the C-tail blocks PTH signaling via PLC. Fluphenazine had no apparent effect on PTH1R signaling through AC (data not shown).

Several lines of evidence suggest that CaM plays a role in PTH1R signaling. In perfused proximal convoluted tubules,

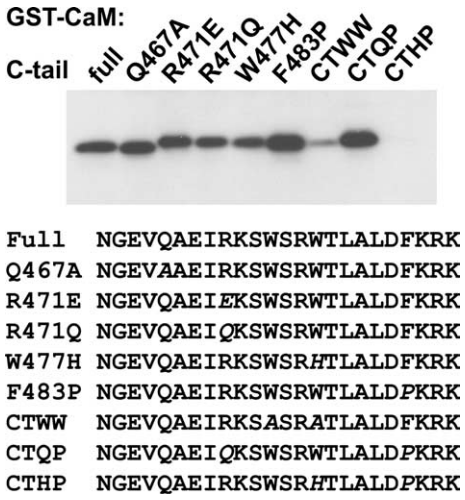

Fig. 4. Mutational analysis of the CaM/PTH1R interaction. The wild type PTH1R C-tail (full) and the C-tail containing the following point mutations, Q467A, R471E, R471Q, W477H, F483P, W474A/W477A (CTWW), R471Q/F483P (CTQP) and W477H/F483P (CTHP), as indicated by italics, were interacted with GST-CaM in the presence of $CaCl_2$. Interactors were eluted and blotted with S-protein-HRP.

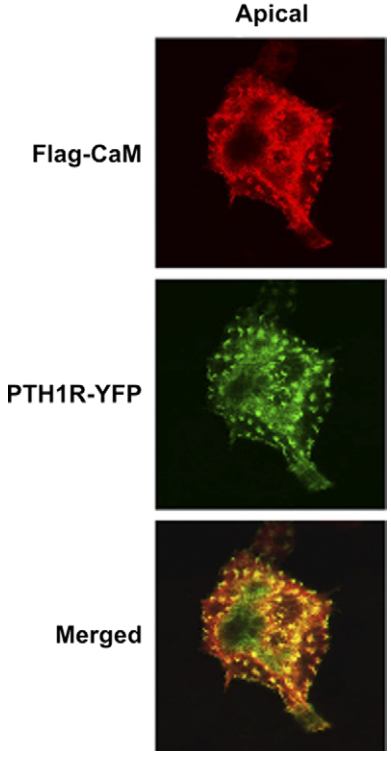

Fig. 5. CaM co-localizes with the PTH1R in apical membranes of OK cells. OK cells were co-transfected with flag-CaM (red) and PTH1R-YFP (green), and stained with flag antibodies and secondary antibodies conjugated with Alexa Fluor 546. Representative confocal images of the apical domain are shown.

PTH inhibited fluid and phosphate transport rates, effects that were abolished by the CaM antagonists, trifluoperazine and W-7 [7]. CaM antagonists also inhibited PTH-induced rarefaction of microvilli in primary cultures of proximal convoluted tubule cells [8]. Furthermore, inhibition of CaM-kinase II

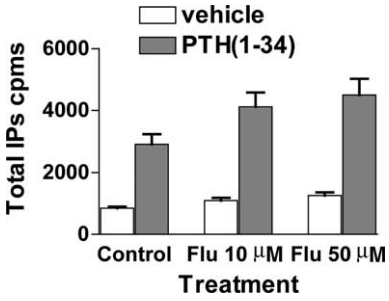

Fig. 6. Fluphenazine enhances PTH1R signaling via PLC. HEK293 cells transiently transfected with the PTH1R were treated with either vehicle (DMSO) or the indicated concentrations of fluphenazine for 15 min. Cells were then treated with PTH(1-34) at 100 nM and accumulation of total inositol phosphates determined. Data are representatives of three independent experiments ($n = 6 \pm$ S.E.M.).

markedly inhibits PTH-induced interstitial collagenase (MMP13) mRNA expression in osteoblastic cells [9]. Lastly, CaM antagonists inhibited PTHrP-mediated production of nitric oxide from endothelial cell primary cultures [10].

CaM regulates GPCR activity via direct interactions. CaM interacts with the μ-opioid receptor via determinants in the third intracellular loop and inhibits high-affinity agonist binding to the receptor by displacing G proteins [11]. CaM also binds to the V2 vasopressin receptor via determinants in the cytoplasmic tail, an interaction that is required for arginine vasopressin-induced elevations of intracellular calcium [12]. Specific isoforms of the group III metabotrophic glutamate receptors interact with CaM via amino-terminal portions of the cytoplasmic tails, an interaction that displaces Gβγ [13,14]. These findings reveal that sequence diversity among the GPCR super-family, even within the same sub-set, provides receptor-specific regulatory elements, such as the CaM-binding motifs, which reflect the function and regulation of a given receptor. For the class b GPCRs, the absence of CaM binding for the secretin and growth hormone releasing hormone receptors suggests that regulation via CaM binding is either not required for normal function or that CaM binding would be deleterious to the normal action of these receptors.

Herein, we describe an interaction domain on the PTH1R cytoplasmic tail that binds to CaM via the basic 1-5-8-14 motif. Notably, this domain is reasonably well conserved among the class b GPCRs, and thus likely represents a common regulatory motif that is responsive to CaM and fluctuations of intracellular Ca^{2+}.

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